THE MEASUREMENT OF THE RESPIRATORY EXCHANGE OF AQUATIC ANIMALS

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Introduction

Recognition of the importance of studying the gas exchange of aquatic animals is attested to by the large number of papers which have appeared on this subject. The present article is the result of a series of investigations on the accuracy of various methods previously described for the measurement of this exchange and presents a method which has proven satisfactory for a rather wide variety of purposes.

Methods of Measuring the Gas Exchange of Aquatic Animals

There are three general types of methods of measuring the respiratory exchange of aquatic animals. The simplest method consists merely in determining the time rate of change in the gas contents of a closed vessel of water containing the animal under investigation. Details and applications of the method are given by Humboldt and Provençal (1809), Henze (1910a), Montuori (1913), Krogh (1916), McClendon (1917), Powers (1922), and Bruce (1926), among many others. The fact that oxygen is diminishing and carbon dioxide and nitrogenous excretions are accumulating in the water during the period of the determination renders this method unsatisfactory. Further objections will appear in the fourth section (" Method finally adopted . . .") of the present paper.

Applications of the Regnault (Regnault et Reiset, 1849) principle to aquatic respiration constitute the second general method. It has been used in various forms by Jolyet et Regnard (1877), Gréhant (1886), Zuntz (1901), Bounhiol (1905), and Gardner and Leetham (1914). The many complications and sources of error in this method have led to criticisms of it by Krogh (1916, p. 50) and Henze (1910b, 1927).

The third method for measuring the gas exchange of aquatic organisms involves a flowing water system. Briefly, a measured amount of water of known gas content is passed, in a given period of time, through a vessel containing the animal, and samples of the water

are collected and analysed after passing through this respiratory chamber. The application of this method has been almost entirely limited to special cases where a sort of artificial respiration is needed, such as in the case of narcotized fish, etc. Thus Winterstein (1908) used it to measure the strictly branchial respiration, while Ege and Krogh (1914) and Gaarder (1918) used it to determine the oxygen uptake of fishes under urethane narcosis. Hall's (1929) use of this method is open to criticism, as I have pointed out elsewhere (Keys, 1930a).

The Investigation of Methods in this Laboratory

Of the three general methods mentioned above, only the first and third were considered. The first method was subjected to a thorough trial in four different arrangements of apparatus. The test animals used in each case were marine fishes (*Fundulus parvipinnis*). A number of difficulties which seemed to be inherent in the method were found. It is not necessary to describe these experiments in detail; one particular source of difficulty was stratification in the gas content of the water in the respiratory vessel.[1]

Having taken into account as many of the sources of error as possible, the final test was made of attempting to duplicate results for the oxygen consumption of individual fishes by this method. In but few of these experiments was the oxygen consumption, as indicated by one determination, duplicated within 15 per cent by a second.

At this juncture experiments with the third method were begun. The apparatus arrangement which had been used by Ege and Krogh was used first. Various modifications of apparatus and technique were subsequently tried until the final method, which has been adopted as standard in this laboratory, was evolved.

It was found that the abstraction of the sample of the incoming water in the manner in which it was done by Ege and Krogh (see Krogh, 1916, p. 51) and, so far as I know, by all others who have used the flowing water method, not infrequently leads to serious errors due to stratification of the water through the respiratory chamber. The most serious drawback to the method of Ege and Krogh was the use of narcotics. Fortunately, however, it was found that the normal, unanesthetized animal could be used without difficulty. A large number of fishes, representing six different species, were observed in small respiratory chambers similar to those used by Gaarder and in every case the fish became perfectly quiet in a few minutes and remained so for many hours so long as a sufficient flow of water was provided.

[1] In some of these earlier experiments I had the assistance of Mr. Rolland Main, whose help is gratefully acknowledged.

Method Finally Adopted for Fishes and Other Macroscopic Aquatic Animals

Figure 1 shows the apparatus used for experiments on the gas exchange of fishes. The respiratory chambers A and B each contain a single fish and are chosen to be of such size that, although almost the whole of the water passing through the chamber comes into contact with
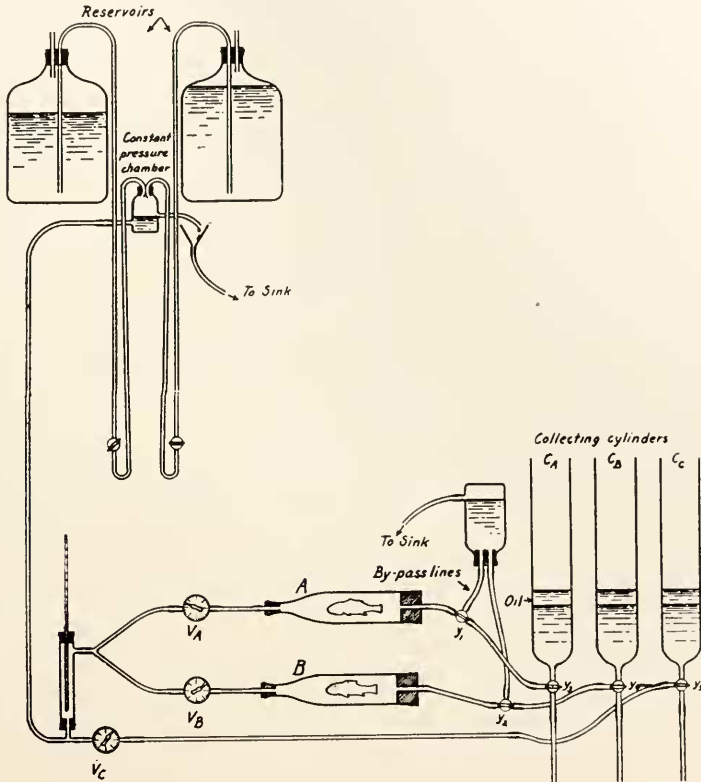

Fig. 1. Apparatus for determining metabolic rates of aquatic animals. The proportionate sizes shown are for the arrangement of the apparatus for studies of the respiratory exchange of small fishes such as *Fundulus*.

A, B, respiratory chambers; Y_{1-5}, 3-way stopcocks; $V_{A, B, C}$, needle valves.

the animal and is available to it for respiration, the fish is not ordinarily in contact with the walls of the chamber and is free to move about to some extent. If the fish is not disturbed after it is placed in the chamber, and if the current of water is properly adjusted at a constant flow, the fish quickly assumes a position facing the incoming stream and thereafter almost perfect quiescence is the rule, the only visible mus-

cular activity being the respiratory movements of the operculum and the leisurely motion of the pectoral fins which serves to keep the fish oriented in the current.

The collecting cylinders C_A and C_B receive the whole of the water which passes through respiratory chambers A and B respectively except when the water is by-passed through the by-pass lines. The by-pass chamber is so designed that it gives approximately the same back-pressure as the collecting cylinders when they are in use and, accordingly, with a given setting of the needle valves V_A, V_B, and V_C, the rate of flow through the system is the same, irrespective of whether the water is being collected for analysis or by-passed. The constant pressure chamber insures the head pressure remaining the same, no matter what the amount of water in the reservoirs. If the collecting cylinders are immovably fixed in position, the back pressure will increase as the cylinders fill, with a resulting decrease in the rate of flow; this can be taken care of by suspending them by long springs or by manually adjusting their level from time to time during each collection.

Collecting cylinder C_C receives the control water for the determination of the gas content of the water before it is respired by the fishes. The possibility of error due to stratification in the reservoirs is thus eliminated by a constant fraction of all the incoming water being diverted to the control. If stratification persists in the collecting cylinders it is easily broken up by gentle shaking or stirring of these last; this, of course, does not disturb the animals in the respiratory chambers.

It will be noted that no provision is shown for maintaining constant temperature. To secure the best results the whole apparatus should be mounted in a constant temperature room. Failing this, the tubing connecting the needle valves with the respiratory chambers should be lengthened into long coils and these last, with the attached respiratory chambers, should be suspended in a constant temperature water bath. In this case the thermometer chamber should be placed inside the bath at the end of one of the coils.

All water for gas analysis should be collected under at least three centimeters of the best quality saturated-hydrocarbon mineral oil. The gas content of the water is readily determined by standard methods, although the determination of carbon dioxide in sea water presents some difficulties. For oxygen the best method is undoubtedly the Winkler (1888) iodometric volumetric method. This method is given in detail in the American Public Health Association manual (3rd edition, 1917), by Treadwell and Hall (1928), and by Sutton (11th

edition, 1924).   The determination of dissolved oxygen in sea water is fully described by Jacobsen (1921).   The determination of carbon dioxide in the water may be made by the Van Slyke manometric method which will be found in the articles by Van Slyke and Stadie (1921), Van Slyke (1927), and Van Slyke and Sendroy (1927).   An alternative method for determining CO_2 in sea water and brackish waters which utilizes pH determinations is given by Bruce (1924).

It should be pointed out that simple modifications, such as in the size of various parts of the apparatus, enable the flowing water method, as presented in this paper, to be used for very small animals.   A correspondingly small volume of water must be used, and in this case the Van Slyke apparatus may be used for both oxygen and CO_2 in water volumes as small as one or two milliliters.   The micro-Winkler method for oxygen (Thompson and Miller, 1928, and Snoke, 1929) may be used with samples as small as five milliliters.   In all determinations, of course, duplicate analyses should be made.
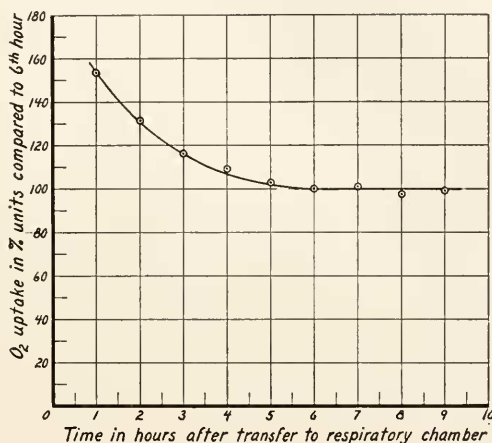

FIG. 2. Serial determinations of the oxygen uptake of *Fundulus parvipinnis,* showing initially high values and the basal or standard rate of exchange attained after several hours. Average curve for ten individuals.

One of the most important considerations in determinations of the gas exchange of the fishes is the time element.   In all experiments reported in the literature the determination was begun as soon as the animal had ceased violent muscular activity, although in some cases even this precaution was omitted.   Usually a single determination was made, the period of this determination varying in different cases from ten minutes to three or four hours; in a very few cases a longer period was

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used. With this in mind I wish to call attention to Fig. 2. The points in this graph were obtained from serial determinations on ten different specimens of *Fundulus parvipinnis*. In order to bring out the general trend in the oxygen consumption of these fishes from hour to hour after transfer to the respiratory chamber, I have reduced all the data to percentage values, taking the oxygen consumption at six hours as the point of 100 per cent. It is clear that a constant rate of oxygen consumption is attained about five or six hours after transfer of the fishes to the respiratory chambers, and that determinations made before this time had elapsed would have overestimated the standard metabolism.

The appearance of the graph for *Fundulus parvipinnis* is characteristic of all the species of fishes which have been studied in this laboratory.[2]

Figure 3 shows an unusually complete series of determinations on a single specimen of *Girella nigricans* and depicts the constancy of results which may be obtained by the use of the method described in this paper.[3] I wish to make it clear that in all cases the fishes were very quiet after the first quarter or half hour in the respiratory chambers. In spite of this fact, the gas exchange did not reach a constant level until from four to six hours. In several hundred of these serial determinations this was found to be invariably the case. The results of a series of determinations on a specimen of *Clinocottus analis* are shown in Fig. 4.

These same general results were obtained in large as well as in small respiratory chambers and were found when the fishes were in total darkness as well as in ordinary daylight. They point to the conclusion that, in order to be sure that the determinations represent " standard " or anything like " basal " metabolism, it is necessary to wait five or six hours after the fish has been placed in the apparatus before beginning the final determinations. These findings throw doubt on the validity of any method in which the period of the determination of the gas exchange begins shortly after the transfer (and handling incident to this) of the animal to the vessel which is used for the experiment.

The procedure recommended in beginning gas exchange experiments on an animal form which has not previously been investigated, is to make the determinations serially, which is very simple with the apparatus described in this paper, until constant gas exchange is reached. Experiments in this laboratory indicate that, in the case of

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[2] These species are *Girella nigricans, Fundulus parvipinnis, Clinocottus analis, Leptocottus armatus.*

[3] I was fortunate in having the collaboration of Mr. N. A. Wells in the application of the method to *Girella* and to *Clinocottus analis.*
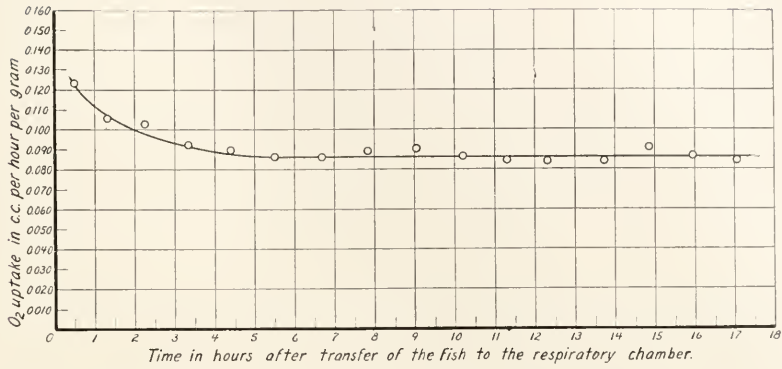
FIG. 3.   Serial determinations of O₂ uptake of *Girella nigricans,* fish no. G₄, weight 40 grams.   Three days starvation.   Temperature 16.7° C.
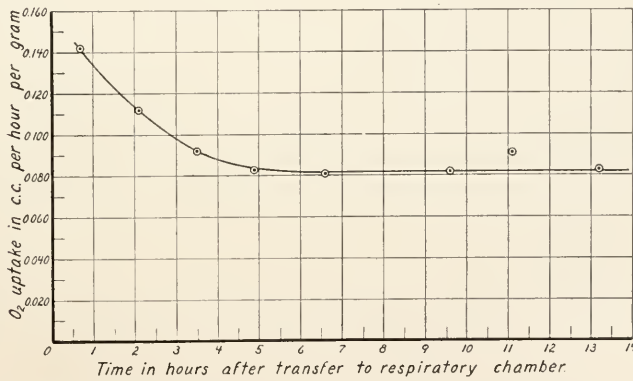

FIG. 4.   Serial determinations of O₂ uptake of *Clinocottus analis,* fish no. C₅, weight 37 grams.   Temperature 20.0° C.

most fishes, it is perfectly safe simply to transfer the animals to the apparatus, allow the system to run (through the by-pass lines) for six hours, and then make two or more determinations covering, let us say, about two hours as a minimum. If these determinations check reasonably well (as is ordinarily the case), it may be assumed that the results (or the mean of the results) represent standard metabolism.

The characteristic appearance of the time graph of the gas exchange demands some comment. The elevated gas exchange which persists for a matter of some hours is not due to muscular activity, at least no such activity is visible. It seems certain that at least part of it is due to the payment of an oxygen debt contracted by the fish in its struggle incident to being placed in the apparatus.[4] Another possible explanation for the phenomenon might be found in the fact that, whereas a certain minimum stimulus is necessary to bring about a nervous impulse and consequent muscular action ("all or none" principle), sub-minimal stimuli will cause a marked increase in the gas exchange of nervous tissue at least (Winterstein and Hirschberg, 1927).

I have already indicated how, by making serial observations, it is a simple matter to demonstrate that the method developed here is capable of yielding very consistent results. In a number of cases determinations of the oxygen uptake of certain fishes were repeated after a lapse of from two or eight days. The results of these experiments are summarized in Table I.

The average discrepancy between first and second determinations is 2.8 per cent. In each case the oxygen uptakes in the table represent the average of two or more determinations. I should warn the reader that such good results are not to be expected until the investigator has had considerable experience with the technique. Good results require unremitting vigilance in the maintenance of constant physical and physiological conditions.

It will be noted that in Fig. 1 there are two respiratory chambers shown. This is a highly desirable feature as it enables the investigator to compare two animals, even though it is not possible to maintain standard conditions. Systematic errors in the determinations are fre-

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[4] The period of payment of oxygen debt is usually not nearly so prolonged in mammals (Hill and Lupton, 1922; Campbell, Douglas and Hobson, 1920; Krogh and Lindhard, 1920) as is indicated in the experiments on fishes but, as Barcroft (1925) points out, the duration of the debt is directly related to the adequacy of the blood supply, and we know (Krogh, 1929) that the capillary blood supply to the muscles of fishes is meagre compared to that of the warm-blooded animals. We should expect the payment of the oxygen debt of any cold-blooded animal to be slower than in a warm-blooded animal in the same way that all the vital processes proceed at a relatively slow rate in the cold-blooded animals.

quently detected if two animals are used in this way. Of course, if the experimenter desires, three, or even more, respiratory chambers with their respective collecting cylinders, valves, etc., may be used, but the services of a number of assistants will be found to be necessary if this is done.

TABLE I

Species	Fish No.	Weight	Date	Temperature	O_2 uptake per hour
		grams		°C.	grams
Fundulus	M_{35}	4.3	12/26/28	20.0	0.207
"	"	"	12/31/28	"	0.214
"	M_{43}	9.1	6/25/29	"	0.161
"	"	"	7/ 2/29	"	0.159
"	M_{44}	4.5	6/25/29	"	0.209
"	"	"	7/ 2/29	"	0.202
"	M_{50}	7.3	6/28/29	"	0.194
"	"	"	6/30/29	"	0.208
"	M_{51}	4.4	6/28/29	"	0.280
"	"	"	6/20/29	"	0.281
Girella	A_7	257	12/ 5/29	20.0	0.0615
"	"	"	12/10/29	"	0.0623
"	A_9	210	12/12/29	18.0	0.0586
"	"	"	12/20/29	"	0.0607

There are a number of precautions which should be taken. The water used should always be filtered and well aërated before use, but should never be supersaturated. The whole system should frequently be tested for leaks and should never be used with air bubbles trapped in any part of the apparatus. The animals used should always be starved long enough before the determination to insure that the digestive tract is empty at the time of the experiment.

Applications of the method described here to particular problems involving a study of the gas exchange of aquatic animals are given in a paper by the author (Keys, 1930b) now in press, as well as in other papers in the course of preparation at the Scripps Institution.

CALCULATION OF RESULTS IN RESPIRATION EXPERIMENTS

The calculations for the determination of oxygen and carbon dioxide by the Van Slyke manometric apparatus are given in the papers of Van Slyke already cited. The calculations for oxygen by the Winkler method are given in the references on this method listed above.

Tables of the normal saturation values of water with respect to oxygen are given by Fox (1907), Jacobsen (1921), and Harvey (1928). Fox's data (given also by Harvey) were obtained by equilibrating water of various salinities with atmospheric air at different temperatures. Jacobsen's data are for air minus water vapor and carbon dioxide. Salinity may be converted into chlorinity by the use of Knudsen's formula

$$Cl = \frac{\text{salinity} - 0.030}{1.8050}$$

where Cl is the chlorine equivalent of the chlorine, bromine and iodine in the sea water, and salinity is the total weight of salts (carbonates as oxides and organic matter ignited) in grams per 1000 grams of sea water.

SUMMARY

The methods previously used for the measurements of the gas exchange of aquatic animals are discussed. Of the three general methods, two were subjected to careful investigation in experiments on marine fishes.

The method finally developed for the study of aquatic respiration is described in detail and typical results obtained by its use are given. It is shown that the method yields consistent results which may be accepted as measures of standard metabolism.

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